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Original Article

Quantitative Assessment and Reduction of Long-term Autoradiographic Background

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Quantitative autoradiography can measure distribution patterns in an animal exposed to radiolabeled compounds. A comparison of autoradiographs of rat brain containing low levels of ^{14}C showed that a highly variable background signal had been produced. This resulted in several overexposed autoradiographs which could not be quantitatively compared. The background, believed to be produced by light emanating from the phosphor coating in the X-ray cassette, was a major impediment because it hindered correct analysis of the specimen. This article details our experiments demonstrating the sources of variance contributing to background and offers methods for its reduction. We found that placement of black polyethylene plastic between the slides and phosphor in the X-ray film cassette minimized autoradiographic background and effectively eliminated the effects caused by inherently different levels of radioactivity in the glass slides. (*J Histochem Cytochem 38:581–583, 1990*)

KEY WORDS: Quantitative; Autoradiography; Phosphor; Background; Variance reduction; Rat; Central nervous system.

Introduction

Quantitative autoradiography provides solutions to several technical problems encountered in the field of biology (1–4). Actions of endogenous and exogenous compounds can be followed through the course of distribution, binding, metabolism, and elimination (2, 9). In addition, the kinetics of these processes and the associated functional implications of compound interactions can be determined (5–7). Track-tracing autoradiography can define submicrometer binding loci (8).

In our work, we used ^{14}C-labeled soman, a powerful organophosphorous anti-cholinesterase (specific activity 56 mCi/mmol) at a subconvulsive dose of 17.3 µg/kg, IM. The objective was to quantitate the kinetics of distribution in the CNS of rats (9). As a result of both the low specific activity of the labeled soman and the extremely low dose, long exposure times were required to produce a readable autoradiographic exposure. We expected soman to accumulate at cholinesterase sites.

We employed image analysis and enhancement techniques to improve signal-to-noise ratios and quantitate results. One problem we encountered was a large variance in background optical density. Our hypothesis was that glass radionuclides were interacting with the autoradiographic film and/or the cassette enhancement phosphor. This report attempts to define the source and the amount of variance and recommends methods for its reduction.

Materials and Methods

Autoradiography. Autoradiographic data were collected from frozen rat brain tissue mounted on glass slides as described elsewhere (9). Three groups of 26 slides each were placed in X-ray film cassettes (Spectroline 8 × 10-inch cassette; Spectronics Corp., Westbury, NY) with the tissue apposed by DuPont Lo Dose mammography film (emulsion side facing tissue) and were exposed for 703 days. These same slides were subsequently re-exposed for 736 days under identical conditions and configurations, with the exception that a black polyethylene sheet 0.005 inches thick was placed between the slide and the phosphor coating on the bottom surface of the cassette. An unexposed control film was also developed to measure film contribution to optical density, generating a total of seven films. The films, including the control, were developed according to standardized methods (7).

Image Acquisition and Analysis. A Hamamatsu Image Acquisition System C1440 (Hamamatsu Systems; Waltham, MA) was used. Images were acquired under non-varying light and camera conditions. High acquisition system sensitivity required elimination of all extraneous light.

All images of slides from both of the 2-year studies, controls, and light source image data were analyzed using a custom program (Image Analysis System, v9.9; DAKKRO Corp, Denver CO). The images were acquired as transmitted light data. Reduction of variance in slide images required the application of corrections derived from analysis of light source and clear film data. The slide image data were divided by control film data for all six samples. The divided images were converted to optical density.

After generating the optical densities for all images, the data were evaluated using a program function of the image analysis system that calculated optical density statistics for user-selected portions of the image. Data were collected horizontally across the non-tissue (glass) portion of each image.

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System response linearity to transmitted light was determined using an optical density wedge.

Two-tailed *t*-tests were used to analyze the data obtained from the images.

Results

The results in Table 1 [images (A) through (F)] clearly show that blocking the light from the phosphor with the plastic essentially eliminated the increased exposure due to phosphor ($p \gg 0.9999$, *t*-test). In images (A) and (B), it normalized the differences in the two types of slides ($p \gg 0.9999$, *t*-test). In addition, there are essential differences in the level of signal generated by the two slide producers (A and C).

Regression analysis of optical wedge data provided the equation OD = 0.0129 + 0.1217(X) with a correlation coefficient of 0.999985.

Statistical analysis of the clear control film yielded the following results: mean OD of 0.4731, standard deviation of 0.0029.

An analysis of signal-to-noise ratios for the various combinations of tissues and slide backgrounds showed ratios varying from 6:1 to 18:1 in the data generated from the cassettes with opaque light block to 0.28:1 to 0.62:1 for cassettes with no opaque blocking.

Discussion

The variation observed from both the measuring system and the autoradiographic configuration showed that small differences in optical density could be reliably measured. The differences in materials used to obtain autoradiographic data, such as the glass slides and type of holding cassette, appeared to be the major source of the observed variation detailed below.

System variance (from camera, electronics, and lens nonlinearities) was present in all of the original images. Images from the light source (LS) and light source plus film (LSF) were used to define this variance and the methods for its reduction. In LS and LSF, there was a non-random fluctuation of transmitted light data values resulting from spherical lens astigmatism, which was correctable. There were other minor non-random signal sources from camera electronics. The percentage variation was 33% for both LS and LSF. Therefore, LSF was used to remove variance due to the system from the subsequently measured slide images by division. This process had no effect on the OD values from non-system errors. Using LSF to normalize the slide images reduced system variance from 33% to 2.7%.

The method described above was able to reduce the amount of system variation in the slide images. However, variation between individual glass slides was still evident. The hypothesis was that radioisotopes from the glass slides interacted with the covering of phosphor used to enhance X-rays in the cassette, thus causing light to be produced. The film in the first 2-year study was exposed to this excess light and produced a higher optical density than the subsequent exposures. To block light from the phosphor, a piece of opaque black polyethylene plastic was placed between the glass slides and phosphor.

Analysis of the results from quantitative optical density analysis of six slide/cassette configurations showed three significant trends. First, Table 1 summarizes the results of the OD measurements and shows that there are major differences between cassettes with and without black plastic between the cassette phosphor and slides. Second, the results show that there are major differences between the levels of background radiation emitted by the slides tested from two different manufacturers. It should be noted that this observation is only valid for these lot numbers of slides and cannot be generalized to all slides produced by these manufacturers. This was not quantitatively analyzed as a part of this study. However, it was confirmed by visually comparing slides from these two companies in previous studies and observing that not all slides were consistently light or dark across producers. Rather, it seemed to vary as a function of the lot numbers and appeared consistent within a lot rather than across lots. Third, the inclusion of the black plastic eliminates the difference between slides in most instances.

Table 1. *Source and quantitative image data*

Image[a]	Slide[b]	N[c]	Mean optical density (OD)	Standard deviation of OD
(A)	C_1	34	0.6202	0.0020
	C_2	39	0.6177	0.0031
	A_1	37	0.5769	0.0024
	A_2	35	0.5749	0.0020
(B)	C_1	36	0.4984	0.0039
	C_2	33	0.4980	0.0023
	A_1	39	0.4923	0.0018
	A_2	34	0.4953	0.0022
(C)	A_3	41	0.5195	0.0016
	A_4	42	0.5153	0.0020
	A_5	40	0.5176	0.0020
	A_6	36	0.5194	0.0020
(D)	A_3	37	0.5794	0.0027
	A_4	37	0.5760	0.0027
	A_5	38	0.5753	0.0036
	A_6	39	0.5739	0.0028
(E)	A_7	38	0.5721	0.0048
	C_3	38	0.6493	0.0052
	A_8	38	0.5729	0.0014
	C_4	38	0.6329	0.0022
(F)	A_7	41	0.4964	0.0019
	C_3	43	0.5122	0.0018
	A_8	39	0.4965	0.0018
	C_4	40	0.5148	0.0015

[a] Images: (A) Cassette E1 developed without black plastic; (B) cassette E1 developed with black plastic; (C) cassette E2 developed with black plastic; (D) cassette E2 developed without black plastic; (E) cassettes E1–B3 developed without black plastic; (F) cassettes E1–B3 developed with black plastic.

[b] A slides from American Scientific; C slides from Corning Glass.

[c] *N*, number of image pixels analyzed.

An analysis of signal-to-noise ratios indicated that fractional ratios were produced by the cassette configuration that contained no light block. Fractional signal-to-noise ratios mean that there is more noise than signal, which in this case is physically observed as tissue absorbing light emitted by the phosphor which has been activated by the glass radionuclides. The tissue is actually less optically dense than the background. This situation produces autoradiographic data that are not related in any simple way to radionuclides in tissues. It is a complex function of differential absorbance of transmitted light and tissue radionuclide emission.

For long-term autoradiographic exposures, the effect of unwanted background radiation can be reduced by using cassettes without

phosphor enhancers and/or phosphor light output blocked by opaque sheets, together with slides of low inherent radioactivity.

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